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Transplantation of Human Amniotic Mesenchymal in the Treatment of Focal Cerebral Ischemia

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Abstract

This study examined the potential of human amniotic mesenchymal (hAMSC) transplantation in repairing neurological deficits in an experimental focal cerebral ischemia model. Following isolation of hAMSC, growth characteristics and surface antigen expression was observed. Butylated hydroxyanisole (BHA) was used to induce the cultured cells into neuron-like cells, identified by immunocytochemistry. The suture model was used to induce focal cerebral ischemia in the rats, which were subsequently randomly divided into experimental and control groups for treatment with BrdU-labeled hAMSCs or PBS, respectively. Neurological deficits were assessed after transplantation using the Neurological Severity Scores, Beam Balance Test, and Elevated Body Swing Test. Eight weeks later rat brain tissue was processed for hematoxylin-eosin staining and BrdU immunohistochemistry, and survival and spatial distribution of transplanted hAMSCs. hAMSCs proliferated in vitro, and neuron specific enolase expressed in neurons and glial fibrillary acidic protein in astrocytes. The focal ischemia model resulted in varying degrees of left limb hemiplegia accompanied by the right side of Horner's sign. When examined 1, 3, 6 and 8 weeks later, significant recovery in the neurological behavior was detected in the rats treated with the hAMSC transplantation compared to the control (P<0.01). BrdU-labeled hAMSCs were concentrated near the graft site and surrounding areas, in some cases migrating towards the ischemic lesion. Local gliosis and lymphocytic infiltration were not detected. hAMSC exhibit great potential for proliferation, and can be induced to differentiate into NSE-expressing neuron-like cells following treatment with BHA. Moreover, hAMSC transplantation may improve neurological symptoms after focal cerebral ischemia.

Keywords: Placenta mesenchymal; Isolation and culture; Focal cerebral ischemia; Transplantation

Introduction

Cerebrovascular injury is one of the three major causes of death and is the leading cause of adult disability. The annual incidence rate in our country is of about 130-300 million, with 60-100 million deaths, and 75% of survivors suffer disabilities of different degrees. Despite the increasing progress in emergency treatment and early rehabilitation in patients with cerebrovascular injury, treatment options for later presenting neurological dysfunction are lacking.

Regenerative medicine and stem cell research has progressed significantly in the 21st century, offering novel routes for treatment of neurological disorders. Mesenchymal stem cells (bone mesenchymal stem cells, BMSCs), unlike hematopoietic stem cells, are present in bone marrow. BMSCs have become a progressive research field in modern biology and medicine. Mesenchymal stem cells (MSCs) are derived from the mesoderm early in development and can be exploited as an ideal source of seed cells, which exhibit the potential to be induced into osteogenic, chondrogenic, and adipogenic cells, or even tendon and adipose tissues [1-4]. MSCs are easy to obtain culture and expand *in vitro* and can be easily induced into designated tissues. Currently, bone marrow-derived MSCs (BMSCs) are widely used. However, the amount of MSCs in bone marrow is extremely low and accounts for about 0.01~0.001% of the bone marrow derived cells [5]. Increasing evidence indicates that MSCs with osteogenic potential can be isolated from a diverse range of tissues, including adipose tissue [6] and perinatal tissues, such as umbilical cord [7], placenta [8,9], umbilical cord blood [10,11], and amniotic fluid [11,12], or even fetal blood, bone marrow, and liver [13-16].

Placenta, a temporary organ, is important to maintain maternal and fetal oxygen and nutrients during embryonic development. The full-term placenta is composed of amnion and chorion, and our previous findings indicate MSCs can be obtained and expanded from both the amnion (amniotic msenchymal, AMSC) and chorion (chorionic msenchymal, CMSC) of placenta (PMSCs) *in vitro,* and the biological characteristics are still well maintained similar to those of BMSCs. In addition, the cell bank of PMSCs can be set up in advance for clinical trials, suggesting PMSCs may have a wide application prospect [18].

In this study we aimed to establish a stable and reliable hAMSC isolation and amplification method *in vitro*. Following induction into neural cells, the hAMSCs were transplanted into the ischemic tissue in rats subjected to focal cerebral ischemia (MCAo). The survival, migration, and differentiation of implanted cells, as well as the recovery of neurological function, was assessed in rats 1-8 weeks later to examine the potential therapeutic benefit of hAMSC derived neuron-like cell transplantation in the treatment of focal cerebral ischemia.

Materials and Methods

Cell culture

Human AMSCs were obtained from normal postpartum placenta. At first, the amnion and villus layer were bluntly separated, and repeatedly washed with D-Hank's solution including double resistant (100 U/ml penicillin, 100 μg/ml streptomycin). After rinsing, amnion was cut into several 1 mm×1 mm pieces with ophthalmic scissors and digested at 37°C water bath for about 30 min with the action of 2.5 g/L trypsin (Gibco). The digestion of amnion was terminated by DMEM containing 5% calf serum and filtered through 200 mesh cell sieve. The filtered productions of amnion were digested again in a 37°C water bath for about 0.5 h with the addition of 1.0 g/L collagens II (Sigma). Subsequent termination and filtrations were done as the described above. Finally, harvested cell suspensions were centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin-streptomycin (Invitrogen, USA). They were then plated in 25-cm² culture flasks at a density of 106 cells per ml and incubated at 37°C with 5% carbon dioxide. Medium was changed every 2 days. When the established adherent cell colonies reached 70% confluence, they were detached with 2.5 g/L trypsin and replated at a ratio of 1:2 in 25 cm² flasks.

Differentiation of hAMSCs

Human AMSCs, second or third generation cells, were plated onto 6-well plates. When they reached 60% confluence, harvested cells were washed with phosphate-buffered saline (PBS). To induce neural differentiation, hAMSCs were incubated with serum-free medium containing DMSO (2%) and BHA (100 μM). Media were changed every 3 days and incubated for 14-21 days. Finally the neural induced cells were confirmed by NSE and GFAP immunofluorescence staining.

Immunofluorescence

Immunofluorescence was performed on hAMSCs cultured for 24 h. Cells were grown to 60% confluence on 6-wells, washed with PBS for three times, fixed in 4% paraformaldehyde for 30min, washed as described above, permeabilized in 0.3% Triton X-100 for 20 min, and then rinsed with PBS three times. Next, cells were blocked with goat serum for 20 min, incubated with the appropriate primary antibody in PBS for 2 h at 37°C, washed with PBS three times, incubated with secondary antibodies in PBS for 30 min at 37°C (in the dark), and then viewed under fluorescence microscopy. The following primary antibodies were used: rabbit anti-human NSE (1: 500, BOSTER), rabbit anti-human GFAP (1: 500, BOSTER). Secondary antibody for immunofluorescence was goat anti-rabbit IgG (1: 500, Sigma).

BrdU labeling and preparation of cell suspension for transplantation

Third generation cells from AMSCs were harvested and plated in 25-cm² culture flasks and 6-wells at a density of 10^5 cells per ml. The cell pellet was resuspended in low-glucose Dulbecco's modified Eagle's medium (L-DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin (100 U/M), 5 ng/ml bFGF, 10 ug/ml BrdU, and then incubated at 37°C with 5% carbon dioxide for 48 h. BrdU-labled AMSCs were centrifuged at 1000 rpm for 10 min, and the cell pellet was resuspended in PBS at 10^6 cells per ul. Finally 5 ul cell suspensions were used for cell transplantations.

Animal model

Healthy male Wistar rats, aged 3-4 months, weighing 250-300 g, were obtained from Schistosomiasis prevention and control center of Jiangsu Province. Briefly, rats were placed in a supine position on the operating table following an intraperitoneal injection of anesthesia of 10% chloral hydrate (4 m1/kg body weight). A blunt dissection of the sternocleidomastoid was made through the middle line neck incision, the carotid artery (CCA) was isolated and further separated into the right external carotid artery (ECA), internal carotid artery (ICA) and the wing jaw artery. The slipknot was left under the ECA and the wing jaw artery, after threading deep in all the arteries. Then the CCA was clamped and a small incision was made in the proximal sidewall of the ECA and a Nylon suture filament (0.24 mm) was inserted and advanced to a depth of about 18.5 ± 0.5 mm away from the CCA bifurcation. The suture was removed after a 2 hours occlusion of the right middle cerebral artery, the ECA was ligated and the skin was sutured.

Animal grouping

Out of 45 rats subjected to focal cerebral ischemia, 13 rats died and 8 rats did not exhibit paralysis of limbs. The remaining 24 rats were randomly divided into 2 groups (n=12 per group).

hAMSC transplantation: Two weeks after MACo, rats were placed in a stereotactic apparatus and Bregma was exposed through median head scalp incision. Coordinates were marked to target the striatum (1 mm before skull, on the left margin of 2.5 mm, and the depth is 4.5-5.5 mm), and 5 ul BrdU-labled AMSCs cell suspension or PBS control was injected into the striatum with Hamilton syringe for 10 min.

Neurological behavior evaluation

Neurological deficit evaluations were carried out prior to the transplantation and 1,3,6,8 weeks after MCAo using Neurological Severity Scores (NSS) (Table 1), Beam Balance Test (BBT) (Table 2), and Elevated Body Swing Test (EBST). For EBST observers should made the record only when the rat head moved more than 10°C to the vertical

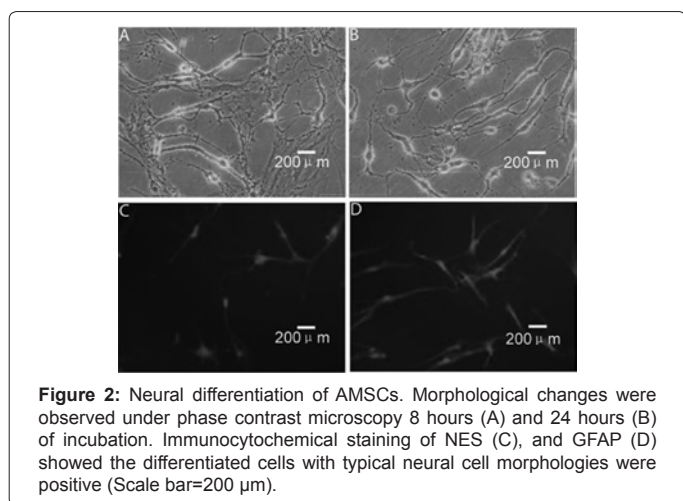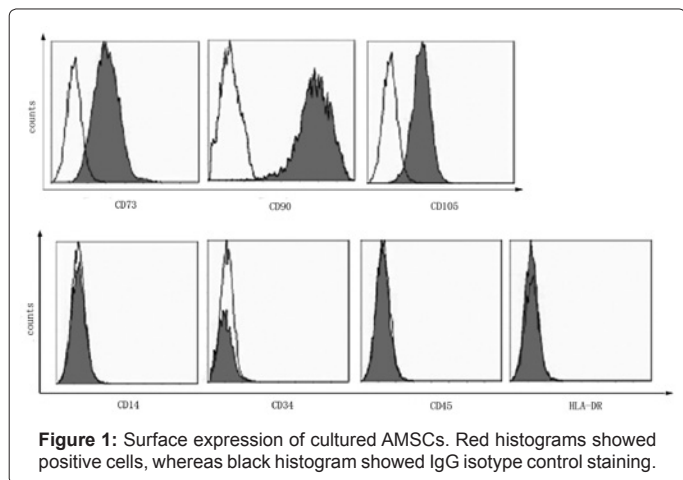| Grading	Point (normal = 0; maximum = 5)
Normal walk	0
Flexion of forelimb (raising the rat by the tail)Q	1
Circling toward the paretic side (walking)	2
Falling down to the paretic side (walking)	3
No spontaneous walking, decreased consciousness	4
Ischemia-related deaths	5

Neurological function in the rats was assessed using the neurological severity scores, the higher the NSS score is, the more severe ischemia will be.

Table 1: Neurological Severity Scores, NSS.

Grading	Point (normal = 0; maximum = 6)
Balances with steady posture	0
Grasps side of beam	1
Hugs the beam and one limb falls down from the beam	2
Hugs the beam and two limbs fall down from the beam, or spins on beam (>60 s)	3
Attempts to balance on the beam but falls off (>40 s)	4
Attempts to balance on the beam but falls off (>20 s)	5
Falls off: no attempt to balance or hang on to the beam (>20 s)	6

Neurological function in the rats was assessed using the Beam Balance Test, The higher the BBT score is, the more severe ischemia will be

Table 2: Beam Balance Test (BBT).

Figure 1: Surface expression of cultured AMSCs. Red histograms showed positive cells, whereas black histogram showed IgG isotype control staining.

Figure 2: Neural differentiation of AMSCs. Morphological changes were observed under phase contrast microscopy 8 hours (A) and 24 hours (B) of incubation. Immunocytochemical staining of NES (C), and GFAP (D) showed the differentiated cells with typical neural cell morphologies were positive (Scale bar=200 μm).

axis within 30' s by raising the rats tail, otherwise, do not record, then 1 min rest, and totally repeated the test 20 times. For the three test above, the records were made on every last day of week 1, week 3, week 6 and week 8, and all rats were tested three times at different time points for each test, taking the average record.

Triphenyltetrazolium chloride (TTC) staining

TTC staining was adopted to show the ischemic area of the brain tissue after 2 hours of perfusion. Then, the brain tissue was coronal cut 2 mm behind the optic chiasm, and the latter part of the brain was immersed into 1% TTC (Sigma-Aldrich) in PBS at 37°C for 30 minutes, and then the 10% neutralized formalin over night.

Preparation of paraffin and frozen sections

Eight weeks after MCAo, the rats were anesthetized intraperitoneally with 400 mg/kg chloral hydrate perfused transcardially with 4% paraformaldehyde in PBS, and brains were quickly extracted. Approximately 2 cm ischemic area of brain tissue, including the lateral ventricles and basal ganglia, striatum and hippocampus was excised, post-fixed in 4% paraformaldehyde, and 5 μm coronal brain slices were consecutively sampled using paraffin sections or frozen sections.

Perl's prussion blue stain for hemosiderin

Sections were transferred through distilled water with xylene and ethanol, placed into the working solution (equal parts mixture

of ferrocyanide and hydrochloric acid) for 15 minutes, rinsed with distilled water and then tap water. Sections were then stained with neutral red for one minute, rinsed well with tap water, dehydrate with ethanol, and finally cleared with xylene. Out of 400 slices every 20th slice was stained using this method to confirm the needle placement and injected sites.

Statistical analysis

All data are presented as mean ± standard deviation. Comparisons of neurological scores were carried out by ANOVA (F test, q test), using SPSS 10.0. The paired t test was used for cell count. In all analyses, a value of $P < 0.05$ was considered significant.

Results

AMSCs culture and cell phenotype

Adherent cells were observed 4 hours after cells were plated, and clone-like growth was observed 48 hours later. The morphology of these cells was similar to that of BMSCs: spindle-shaped with fibroblast-like colonies adhering to the plastic surface. Flow analysis (Figure 1) showed that AMSCs expressed the typical MSC markers (CD73, CD105, CD90) but was negative for hematopoietic markers (CD34, CD45), the monocytic marker (CD14) and also negative for HLA-DR. A large number of BrdU positive cells were observed using fluorescence microscopy, suggesting successful transplantation of AMSCs.
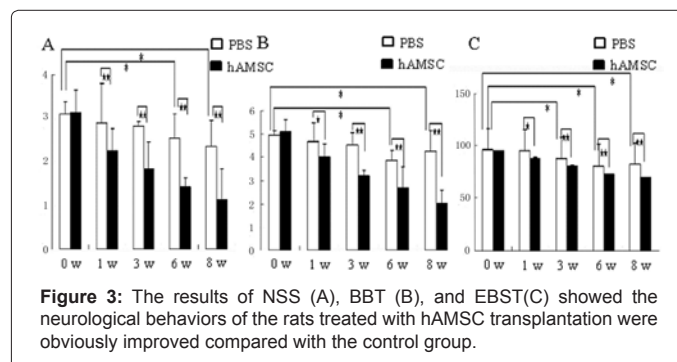
Neural induction of AMSCs

Morphological changes, including condensed cell bodies with outgrowth of a few processes, were detected in some of the cells 2 hours after incubation, (Figure 2A) with more cells showing these neural cell-like changes one hour later (Figure 2B). In addition to the morphological changes, differentiated cells expressed the NES, a marker for neural progenitor cells, and GFAP, a marker for astrocytes (Figures 2C and 2D) respectively.

Neurological function score

All the rats were tested for neurological function at different time points (Figure 3) using the test of NSS (A), BBT (B), and EBST(C). For each test the neurological behaviors were markedly improved, and there was a significant difference between the AMSCs transplanted group and the PBS injected groups.

TTC staining and HE staining

TTC staining has been a standard for the measurement of infarct size and has been used previously for assessment of infarct size resulting from apoptosis and necrosis [6]. Normal brain tissue was showed in red color while the ischemic area was white in TTC staining (Figure
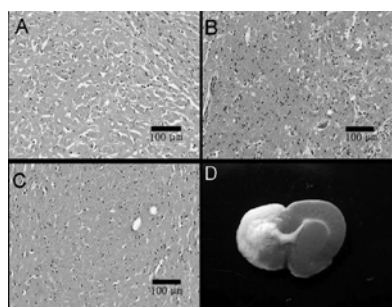

Figure 3: The results of NSS (A), BBT (B), and EBST(C) showed the neurological behaviors of the rats treated with hAMSC transplantation were obviously improved compared with the control group.

Figure 4: Histology of MCAo area in the rats, on the 8th week after transplantation. (A) HE staining showed the normal brain tissue of the rats. (B) The ischemic area of the brain tissue from the control group (PBS injected group). (C) The ischemic area of the brain tissue from the experimental group (AMSCs transplanted group). (D) TTC staining showed the ischemic area of the brain tissue (Scale bar=100 μm, in A, B, C).
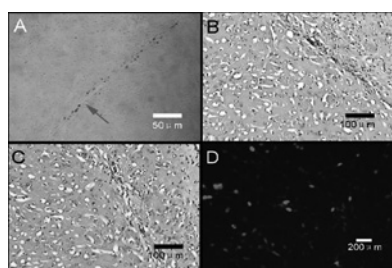

Figure 5: On the 8th week after transplantation, the injected sites and needle passage was showed by hemosiderin staining (the specific place was indicated by the red arrow, Scale bar=50 μm) (A). More lymphocytic infiltration and glial cell proliferation were found around the needle passage for the PBS injected group (B) than in the AMSCs transplanted group (C), (HE staining, Scale bar=100 μm). (D) Under the fluorescence microscopy, the Brdu positive AMSCs were found distributed around the needle passages (Scale bar=200 μm).

4D), and the ischemic region was observed in the dorsolateral striatum and the lateral of the ischemic hemisphere, which is consistent to the blood supply area of middle cerebral artery (Figure 4A). In the ischemic hemisphere, HE staining indicated a large necrotic area on the 8th week after transplantation. Within this region there was a significant loss of neurons, with only a few remaining astrocytes, and a marked interstitial edema. The surviving neurons had varying degrees of morphological changes, with the most obvious changes occurring in pyramidal cells, which showed a shrunken cell body, retracted processes, and loss of Nissl bodies. Furthermore, the chromatin became cloudy and the nuclear membrane shrunk., which was called as 'Pink shrink-like cells', or 'Black contraction-like cells' (Figure 4B). Fewer degenerated cells were observed in the AMSCs transplanted area (Figure 4C).

Determination of injection sites, needle passage and the BrdU-labeled transplanted AMSCs

The injection sites and needle tracks were identified in each of the 24 rats using specific hemosiderin staining (Figure 5A), showing that 17 were injected into the striatum, and the other 7 in cerebral cortex. A small amount of lymphocytic infiltration and a small glial cell proliferation were observed around the needle tracks (Figures 5B and 5C). In the experimental group, BrdU-positive AMSCs - near the ischemic lesion were found distributed around the needle passages (Figure 5D), with some cells residing up to 2 mm away. No BrdU positive cells were observed around the needle passages in the control group.

Discussion

Cell replacement therapy has recently become a developing and promising approach for treatment of central nervous system injury and disease. In this study, we use the focal cerebral ischemia model in rats and implanted hAMSC to the ischemic hemisphere using stereotaxic targeting to the striatum or cortex. We observed cell survival and differentiation -of hPMSCs in cerebral ischemia rat brain, associated with recovery of neurological function. We found that PMSCs implanted into ischemic tissue in rats improved neurological function and balance beam tests relative to the control group. Similarly, histological staining test showed PMSCs survival within the ischemic region.

Silva et al. analyzed gene expression of MSCs and found that MSCs not only code the genes of mesenchymal tissue, but also the genes of endothelial and epithelial tissues [18]. These results provide a theoretical basis of potential differentiation of MSCs. MSCs can be used to replace a variety of cells due to their inherent plasticity of cross-system and even cross germ layer differentiation. Jie et al., showed that bone marrow MSCs of rats spontaneously express neural specific proteins [19], such as NES, ß-III tubulin, NFM, S100-ß. In this study, NSE and GFAP were detected expressed after the hAMSCs was induced by BHA furthermore, placental amnion developed from embryonic ectoderm so we speculated the amniotic MSCs can be easily induced to differentiate into astrocytes and neuronal cells compared to the MSC derived from other sources. Therefore, hAMSCs have broad application prospects in the treatment of nervous system damage and repair cell research.

When determining the best transplantation time points of hAMSC following ischemic injury, it is important to consider both the release of toxic neurotransmitters and oxygen free radicals, at early stage of transplantation, and influence of scar formation on the growth and differentiation of transplanted cells at chronic infarction. For example, Li et al. found that when cells were transplanted 1d or 7d after acute stroke, nerve toxins, free radicals and release of proinflammatory mediators would lead to the further development of ischemic injury adversely and affected the transplanted cells underwent apoptotic cell death in the ischemic penumbra [21]. In addition, inflammation can activate microglia and inhibit the growth and survival rate of endogenous neural cells. According to Fukunnaga et al., the best treatment window of BMSCs transplantation was at least 1 month after stroke [22]. Here, we transplanted cells two weeks after stroke and found that hAMSC are dense within the ischemic lesion, suggesting they migrate and/or proliferate within injured tissue. Furthermore, we found that 8 weeks after cell transplantation, neurological function was improved compared to the control group.

In this study we showed that transplantation of hAMSC markedly improves neurological recovery following MCAo through stereotaxic injection, and the recovery probably associated with the secretion function of implanted MSCs, as it was reported the ratio of cell survival and differentiation reaches about 80% *in vitro* [23] and only 3%-10% *in vivo* [24]. Overall the mechanism underlying recovery is yet unclear and warrants further investigation. However, the approach we describe in this study offers a promising new route for treatment of neurological disorders, including ischemic stroke.

References

1. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, et al. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418: 41-49.

2. Kadiyala S, Young RG, Thiede MA, Bruder SP (1997) Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant 6: 125-134.

3. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, et al. (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98: 2615-2625.

4. Vaananen HK (2005) Mesenchymal stem cells. Ann Med 37: 469-479.

5. Rao MS, Mattson MP (2001) Stem cells and aging: expanding the possibilities. Mech Ageing Dev 122: 713-734.

6. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211-228.

7. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE (2005) Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 23: 220-229.

8. Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, et al. (2008) Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. 26: 300-311.

9. Soncini M, Vertua E, Gibelli L, Zorzi F, Denegri M, et al. (2007) Isolation and characterization of mesenchymal cells from human fetal membranes. J Tissue Eng Regen Med 1: 296-305.

10. Bieback K, Kern S, Kluter H, Eichler H (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22: 625-634.

11. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, et al. (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103: 1669-1675.

12. De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, et al. (2007) Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 25: 100-106.

13. Chan J, O'Donoghue K, Gavina M, Torrente Y, Kennea N, et al. (2006) Galectin-1 induces skeletal muscle differentiation in human fetal mesenchymal stem cells and increases muscle regeneration. Stem Cells 24: 1879-1891.

14. Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, et al. (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98: 2396-2402.

15. Chan J, O'Donoghue K, de la Fuente J, Roberts IA, Kumar S, et al. (2005) Human fetal mesenchymal stem cells as vehicles for gene delivery. Stem Cells 23: 93-102.

16. Chan J, Waddington SN, O'Donoghue K, Kurata H, Guillot PV, et al. (2007) Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse. Stem Cells 25: 875-884.

17. Miao Z, Jin J, Chen L, Zhu J, Huang W , et al. (2006) Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. Cell Biol Int 30: 681-687.

18. Silva GV, Litovsky S, Assad JA, Sousa AL, Martin BJ, et al. (2005) Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 111: 150-156.

19. Deng J, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED (2006) Mesenchymal Stem Cells Spontaneously Express Neural Proteins in Culture and Are Neurogenic after Transplantation. Stem cells 24: 1054-1064.

20. Li Y, Chopp M, Chen J, Wang L, Gautam SC, et al. (2000) Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. J Cereb Blood Flow Metab 20: 1311-1319.

21. Fukunaga A, Uchida K, Hara K, Kuroshima Y, Kawase T (1999) Differentiation and angiogenesis of central nervous system stem cells implanted with mesenchyme into ischemic rat brain. Cell Transplant 8: 435-441.

22. Jori FP, Napolitano MA, Melone MA, Cipollaro M, Cascino A, et al. (2005) Molecular pathways involved in neural in vitro differentiation of marrow stromal stem cells. J Cellular Biochem 94: 645-655.

23. McCully JD, Wakiyama H, Hsieh YJ, Jones M, Levitsky S (2004) Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 286: H1923-H1935.